Reference: *Biol. Bull.* **168**: 175–182. (February, 1985)

HEMAGGLUTININ AND HEMOLYSIN LEVELS IN THE COELOMIC FLUID FROM *HOLOTHURIA POLII* (ECHINODERMATA) FOLLOWING SHEEP ERYTHROCYTE INJECTION

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Abstract

After injection of formalinized sheep erythrocytes into the coelomic cavity of *Holothuria polii* the activity of the naturally occurring hemagglutinins remained constant, while the hemolysin level rose over an eight day period. The kinetics of the response were the same after a further injection, although the hemolytic titers reached higher levels over a longer period. Results obtained using rabbit erythrocytes indicate that this response can be considered a secondary one: higher titers were demonstrated over a 24 h period. Some properties of both naturally occurring and induced hemolysins are discussed.

Introduction

Coelomic fluid from the polian vesicles of *Holothuria polii* shows hemagglutinating and hemolytic activity against a variety of erythrocyte types. Studies of anti-rabbit erythrocyte hemagglutinin and hemolysin have shown that they are proteins with differing chemico-physical properties.

Hemagglutinin is relatively heat stable (85°C) and sensitive to low pH and high ionic strength; its activity is independent of divalent cations (Ca^{2+} or Mg^{2+}) (Parrinello *et al.*, 1976). Hemolysin is a thermolabile molecule which lyses erythrocytes in alcaline medium supplemented with Ca^{2+} (Parrinello *et al.*, 1979). Further differences concern molecular weight and subunit organization (Canicattì and Parrinello, 1983).

Since these proteins may be involved in holothurian internal defense, as reported in other invertebrates (McKay *et al.*, 1970; Cooper *et al.*, 1974; Stein *et al.*, 1981, 1982), the possibility of increasing the hemagglutinating and hemolytic activity of the coelomic fluid was investigated by means of erythrocyte injections. Some properties of the hemolysins are also reported.

Materials and Methods

Adult *Holothuria polii* were collected from the Gulf of Trapani and maintained at 15°C in running sea water. The coelomic fluid was obtained by intracoelomic puncture and from the polian vesicles, pooled, and centrifuged at 400 × *g* for 30 min at 4°C to remove cells. The supernatant was stored at −75°C.

To prevent lysis, sheep erythrocytes were formalinized according to Csizmas's method (1960). After several washings with phosphate buffered saline at pH 7.4 (PBS),

Received 6 August 1984; accepted 20 November 1984.

Abbreviations: SE = sheep erythrocytes; RE = rabbit erythrocytes; HE = human ABO erythrocytes; fSE = formalinized sheep erythrocytes; CF = coelomic fluid from the body cavity; PVCF = coelomic fluid from the polian vesicles; PBS = phosphate buffered saline (0.01 *M* pH 7.4 phosphate buffer containing 0.15 *M* NaCl); EDTA = ethylenediaminetetra-acetic acid.

packed formalinized sheep erythrocytes (fSE) were suspended in PBS at a concentration of 6×10^8 cells/ml. 0.15 to 0.20 ml of this suspension was injected into the coelomic cavity. Animals injected with 0.15–0.20 ml PBS were used as control. To study the secondary response a second series of injections were given 11 days after the first one.

Hemagglutinin titers and degree of hemolysis were evaluated as previously described (Parrinello *et al.*, 1976, 1979). Injected specimens (5–10) were sacrificed daily, and the coelomic fluids from the body cavity and polian vesicles were separately pooled and tested with sheep (SE), rabbit (RE), and human ABO (HE) erythrocytes. Unless otherwise specified, the coelomic fluid tested was from the body cavity. To absorb the hemolytic activity, packed formalinized RE or SE, washed several times with Tris 0.05 *M*–NaCl 0.15 *M*, were added to an equal volume of coelomic fluid previously dialyzed against Tris–NaCl–$CaCl_2$ 0.02 *M*. After incubation for 1 h at 37°C followed by 12 h at 4°C the mixture was centrifuged and the supernatant used for hemolytic assays.

The inhibitory effect of carbohydrates was tested by adding coelomic fluid (0.2 ml) to an equal volume of 2-fold serial dilutions of 0.004 *M* saccharide solution. After incubation for 30 min at 37°C, 0.2 ml of erythrocyte suspension was added. D-galactose, D-glucose, D-fucose, L-fucose, D-xilose, L-xilose, D-glucosamine, D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-mannose, melezitose, melibiose, cellobiose, α-lactose, sucrose, and raffinose (Sigma) were used.

The Folin-Ciocalteau method as described by Lowry *et al.* (1970) was used for protein content determination. Bovine serum albumin was the reference standard. Each value was expressed as the mean of three determination ± S.E.

Coelomic fluid was fractionated by gel filtration utilizing Bio-gel A5m (Bio-Rad) column (0.9 × 60 cm) equilibrated with PBS. Samples were concentrated by ultrafiltration in a Diaflo equipped with a UM 2 membrane (Amicon Corp., Lexington, MA) and dialyzed overnight against the starting buffer. The fractions were monitored for UV absorbancy at 280 nm and tested for hemagglutinating and hemolytic activities.

Results

Hemolytic and hemagglutinating activity following primary and secondary sheep erythrocyte injections

After the injection of the formalinized sheep erythrocytes the anti-SE hemagglutinating activity of the coelomic fluid was the same as that of controls, whereas the hemolysin titer rose, reaching its highest value (1:8), twice that of the controls (1:2), after eight days. The hemolytic activity dropped back near the control level on the tenth day (Fig. 1, CF).

On the first day the undiluted coelomic fluid samples showed a degree of hemolysis (14–43%) lower than that found in the controls (80–90%). This value increased steadily reaching over 80% on the third day. The highest degree of hemolysis (90%) was found on the fourth day (Fig. 2).

To study the secondary response, a further dose of formalinized sheep erythrocytes was injected 11 days after the first one. No differences were found in the kinetics of the response when compared to the primary one, although the hemolytic titers reached higher levels over a longer period (Fig. 1).

In assays of the undiluted samples after the secondary injection, the degree of hemolysis reached the same levels (80–90%) as the controls throughout the period (Fig. 2).

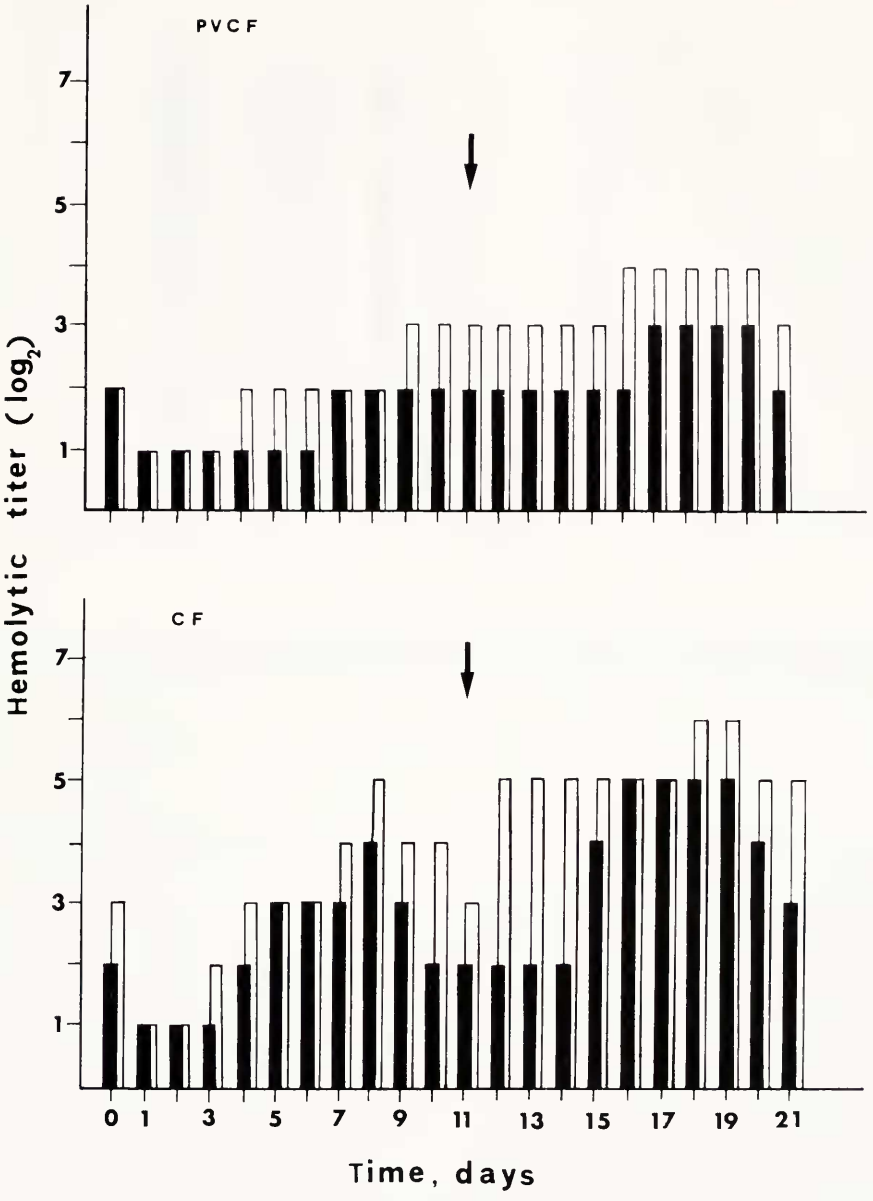

FIGURE 1. Hemolysin levels in the polian vesicle coelomic fluid (PVCF) and body cavity coelomic fluid (CF) of *Holothuria polii* following two injections of 0.15–0.20 ml of 6×10^8/ml formalinized sheep erythrocytes. The diagrams show titers measured with sheep ■ and rabbit □ erythrocytes. The end titer was the reciprocal of the highest dilution revealing hemolysis (at least 10%). The arrows indicate the second injection point.

The hemolysin levels in the coelomic fluid from the polian vesicles were different. Titers increased slightly after the primary and secondary challenges, and in comparison with the coelomic fluid from the body cavity, lower titers were observed over a longer period (Fig. 1, PVCF).

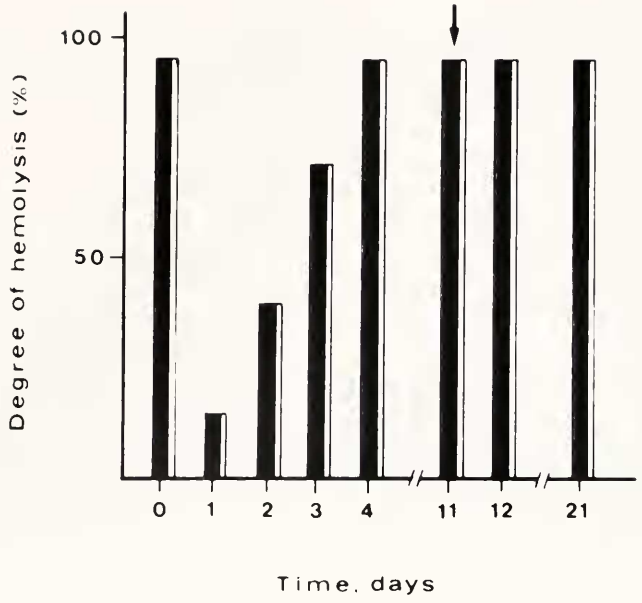

FIGURE 2. Degree of hemolysis of undiluted coelomic fluid following primary and secondary injection of formalinized sheep erythrocytes. ■ = sheep erythrocytes; □ = rabbit erythrocytes. Mean values, S.E. < 1.46, n = 3.

Specificity of the hemolytic response and saccharide inhibition experiments

Each immune sample was assayed with rabbit erythrocytes. The samples obtained seven days after the primary and secondary injection were also tested with ABO human erythrocytes. Increased hemolytic titers were obtained in both cases.

The anti-RE activity varied in the same way as the anti-SE in the period following the first injection; it increased sharply 24 hours after the second fSE injection (Fig. 1, CF).

The titer of hemolysin against ABO-HE was 1:4 in the control coelomic fluid and increased to 1:16 in the immune samples.

Absorption experiments were carried out by mixing (v/v) coelomic fluid with formalinized sheep or rabbit erythrocyte suspensions. The supernatants obtained by centrifuging the reaction mixtures did not show anti-SE, anti-RE, or anti-HE activity.

In attempts to identify the membrane components which react with hemolysin, inhibition experiments with saccharides were performed. None of the sugars used exerted inhibitory activity on hemolysin of immune or control samples. The same results were obtained when the reaction mixtures containing sugar were tested for hemagglutinating activity.

Gel filtration of the immune coelomic fluid

The coelomic fluid obtained after the first and second fSE injections were separately pooled and compared with control coelomic fluid by chromatographic separation. The elution patterns were similar although the hemolysin fraction (third peak) from the immune coelomic fluid showed the greatest UV absorbances, corresponding to increased anti-RE hemolytic titers (Fig. 3). Anti-SE activity in the third peak was

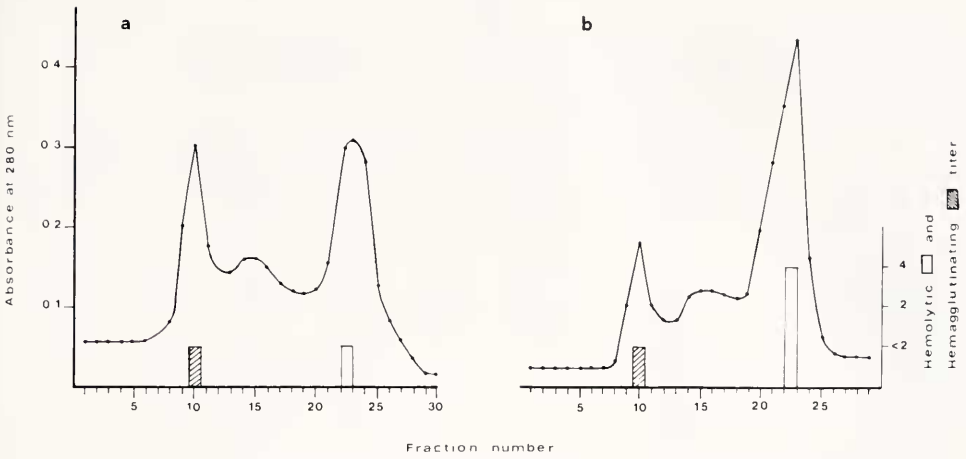

FIGURE 3. Bio-gel A5m elution patterns of *Holothuria polii* coelomic fluid from animals injected with phosphate buffered saline (a) or formalinized sheep erythrocytes (b). Bars indicate the distribution of hemolytic □ and hemagglutinating ▨ activities against rabbit erythrocytes. The end titer was the reciprocal of the last dilution revealing a clear agglutination, or hemolysis (at least 10%).

found only when the pooled fractions were concentrated by ultrafiltration to a fifth of their volume. The titer was higher (1:8) in the immune than (1:2) in the non-immune coelomic fluid; quantification also showed higher protein content (0.314 ± 0.5 mg/ml) of the pooled active fractions in comparison with the corresponding peak of the non-immune samples (0.110 ± 0.5 mg/ml).

Temperature treatments

The anti-RE hemolytic activity of the non-immune coelomic fluid disappeared after heating at 56°C, while a residual activity was maintained against SE until 100°C. The same treatments were carried out on samples from specimens immunized with a second fSE injection. As shown in Table I, the heat stable fraction increased after this injection and also reacted with RE.

In the coelomic fluid from polian vesicles the heat stable fraction was evident only when immune-samples were tested; however, a low degree of hemolysis was also found (Table I).

Role of divalent cations

While the hemolytic activity of the non-immune coelomic fluid was reduced following EDTA (8 m*M*, pH 8) dialysis, a residual activity (40%) was found in the immune samples.

Characteristics of the hemolytic reaction

Some properties of the immune coelomic fluid obtained following a secondary injection were compared to those of the control samples.

To evaluate the reaction time, fixed amounts of coelomic fluid were incubated with a constant RE suspension (6×10^8 cells/ml) and the degree of hemolysis estimated at different times. The reaction of the naturally occurring and induced hemolysin

TABLE I

Effect of temperature treatments on naturally occurring and induced hemolytic activity of Holothuria polii *coelomic fluid*

Samples	Erythrocytes used for the hemolytic reaction	Degree of hemolysis (%) after temperature treatments[1]					
		37°C	56°C	70°C	80°C	90°C	100°C
CF[2] from control animals	RE	87	16	14	14	14	10
	SE	84	51	46	40	40	40
CF from injected animals[3]	RE	94	55	53	50	42	36
	SE	94	84	84	84	80	68
PVCF[4] from control animals	RE	18	2				
	SE	2					
PVCF from injected animals[3]	RE	75	2				
	SE	25	25	23	ND		

[1] Mean values, S.E. < 1.7, n = 3.
[2] CF = coelomic fluid from the body cavity.
[3] The samples were obtained after a second stimulation with formalinized sheep erythrocytes.
[4] PVCF = coelomic fluid from the polian vesicles.
RE = rabbit erythrocytes; SE = sheep erythrocytes.

was very fast; after 5 minutes the degree of hemolysis rose to 60% and its highest value (94%) was reached after 20 minutes.

To determine the amount of the immune coelomic fluid required to lyse a constant number (6×10^8 cells/ml) of RE, sample scalar dilutions were incubated with erythrocytes. The curve presented in Figure 4 shows that from 15 to 80% there is a linear relationship between degree of hemolysis and amount of coelomic fluid while, as the degree of hemolysis approaches 0 or 100%, large increases in coelomic fluid result in smaller increases in the degree of hemolysis. Similar behavior and curve shape were found when HE (6×10^8 cells/ml) were used.

DISCUSSION

The results show that the hemolytic system of *Holothuria polii,* when examined by hemolysin titration, responded in three phases: an initial decrease, probably due to the involvement of the hemolysins in the clearance of foreign materials; an increase, which could depend on stimulation and differentiation of the producer cells; and a final decrease to the level of the controls. This stimulation of the hemolytic system is also indicated by the gradual increase, after an initial reduction, in the degree of hemolysis in the undiluted samples collected over the whole response period reaching maximum before the titer values increase.

A further injection of the same erythrocytes in the third phase of the primary response produced a similar pattern, so far as the interval between injection and titer increase are concerned, although its magnitude changed. There was no first phase decrease in titers and hemolysin levels, the former subsequently reaching values which were significantly higher than those of both the controls and the primary response.

The induction of the coelomic fluid hemolytic fraction was also shown by the quantification of the third peak in the elution chromatographic pattern of the immune sample.

The hemolytic system of *H. polii* therefore seems to be primed after the first injection, and the response to a further injection can also be considered a secondary

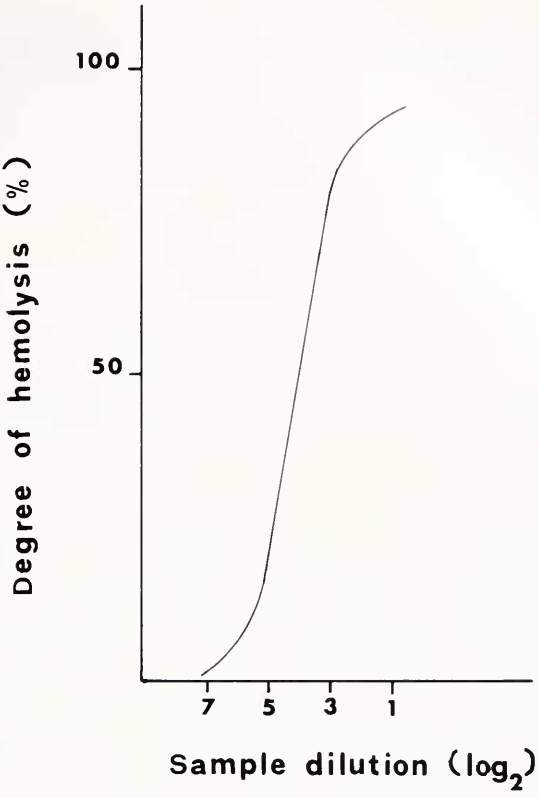

FIGURE 4. Relationship between concentrations of coelomic fluid from animals injected with formalinized sheep erythrocytes, and degree of hemolysis of a constant number (6×10^8 cells/ml) of rabbit erythrocytes. Mean values, S.E. < 2.5, n = 3.

one on the basis of the results obtained using rabbit erythrocytes as a test system. These red cells are more sensitive to hemolysis by *H. polii* coelomic fluid (Parrinello *et al.*, 1979) and, as shown by absorption studies, their receptors are recognized as cross-reactive by anti-SE hemolysin. Thus the secondary response appears faster than the primary one, and high titers are achieved after 24 hours. We do not know if this is a specific response because the induced hemolysin also reacts with the other erythrocytes used in this study (HE) probably due to its wide specificity range.

The hemolytic reaction is very fast. As indicated by the dose-response curve, several factors could be involved in the hemolytic activity of the coelomic fluid. The sigmoidal shape of the curve could be explained by a model requiring various components acting together to lyse the erythrocyte. The possibility that the multiple binding of a component is required for lysis cannot be excluded.

The coelomic fluid contains heat-sensitive and heat-stable hemolysins, of which the latter lyses SE, and, as suggested by absorption experiments, it is able to link up with the RE surface. The heat-stable hemolysin has been induced by fSE injections; it is also able to lyse RE. As shown by EDTA treatments, the activity of the induced fraction could be independent from divalent cations. Further investigation is needed to clarify the differences between these two components of the *H. polii* hemolytic system, and their relationships with complement factors should be studied.

The starting mechanism of the lytic reaction seems to exclude membrane receptors characterized by sugars, which in this study are used for competitive inhibition experiments.

The production of hemagglutinins in the coelomic fluid of *H. polii* was not stimulated by the formalinized erythrocyte injections. However, the dose and/or formalinization of the erythrocytes could influence the strength of the holothurian response.

ACKNOWLEDGMENT

This work was supported by an M.P.I. grant.

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